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Generation and Comparative Kinetic Analysis of New Glycosynthase Mutants from *Streptococcus pyogenes* Endoglycosidases for Antibody Glycoengineering

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Abstract

Chemoenzymatic glycan remodeling by endoglycosidase-catalyzed deglycosylation and reglycosylation is emerging as an attractive approach for producing homogeneous glycoforms of antibodies, and the success of this approach depends on the discovery of efficient endoglycosidases and their glycosynthase mutants. We report in this paper a systematic site-directed mutagenesis of an endoglycosidase from *Streptococcus pyogenes* (Endo-S) at the critical Asp-233 (D233) site and evaluation of the hydrolysis and trans- glycosylation activities of the resulting mutants. We found that in addition to the previously identified D233A and D233Q mutants of Endo-S, most of the Asp-233 mutants discovered here were also glycosynthases that demonstrated glycosylation activity using glycan oxazoline as the donor substrate with diminished hydrolytic activity. The glycosynthase activity of the resultant mutants varied significantly depending on the nature of the amino acid substituents. Among them, the D233M mutant was identified as the most efficient glycosynthase variant with the highest transglycosylation/ hydrolysis ratio, which is similar to the recently reported D184M mutant of Endo-S2, another *S. pyogenes* endoglycosidase. Kinetic studies of the D233M and D233A mutants of Endo-S, as well as glycosynthase mutants D184M and D184A of Endo-S2, indicated that the enhanced catalytic efficacy of the Asp-to-Met mutants of both enzymes was mainly due to an increased turnover number (increased k_{cat}) for the glycan oxazoline substrate and the significantly enhanced substrate affinity (as judged by the reduced K_M value) for the antibody acceptor.

Graphical abstract

Enzyme	Glycan oxazoline		Antibody	
Endo-S2	k_{cat} (min ⁻¹)	K_M (μ M)	k_{cat} (min ⁻¹)	K_M (μ M)
D184M	168	229	30	32
D184A	10	121	--	>680

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Therapeutic monoclonal antibodies (mAbs) represent a rapidly expanding class of biologics that are widely used for the treatment of cancer, inflammation, and infectious diseases.^{1–3} Previous studies have shown that glycosylation affects a wide range of properties of antibodies, including their structural integrity, serum half-life, effector functions, and therapeutic efficacy.⁴ For instance, a lack of core fucosylation on IgG Fc N-glycans at Asn-297 can significantly enhance antibody-dependent cellular cytotoxicity (ADCC), and the engineered antibody therapeutics with a low fucose content showed substantially enhanced therapeutic efficacy versus those of their fucosylated counterparts.^{5–10} Moreover, a minor sialylated component of intravenous immunoglobulin (IVIG) has demonstrated anti-inflammatory effects in animal models.^{11–13} Nevertheless, antibody preparations from natural resources or recombinant protein expression typically contain a heterogeneous mixture of glycan structures and are not usually optimized for structure–function studies or their therapeutic purposes. For example, only a small percentage of antibody therapeutics carries nonfucosylated N-glycoforms that are most effective for their functions as anticancer drugs.^{14,15} Monoclonal antibodies carrying homogeneous glycan structures become essential materials for studying antibody glycosylation and for improving the therapeutic outcome of antibody-based drugs.

Tremendous effort has been spent to optimize antibody glycosylation through engineering of the glycosylation biosynthetic pathway in various host expression systems.^{16–18} Nevertheless, complete control of the glycosylation profile by host expression engineering remains a challenge, and the glycoforms that can be accessed this way are limited.^{16–18} An alternative approach to circumvent the heterogeneity of antibody glycosylation is to perform *in vitro* chemoenzymatic glycan remodeling using an endoglycosidase-catalyzed deglycosylation and glycosynthase-catalyzed reglycosylation protocol.^{19–22} In this method, heterogeneous N-glycans of the antibody are released by the wild-type endoglycosidases, leaving only the innermost GlcNAc or Fuc α 1,6GlcNAc residue intact on the antibody backbone. Then, the well-defined glycan structures can be reattached to the GlcNAc- or Fuc α 1,6GlcNAc-containing antibody by an endoglycosidase or an endoglycosynthase mutant in a site-specific manner to produce an antibody with homogeneous glycoforms. In 2012, we created two endoglycosynthase mutants, D233A and D233Q, of an endoglycosidase from *Streptococcus pyogenes* (Endo-S) that were able to transfer complex-type N-glycans to the deglycosylated rituximab, which represents the first endoglycosynthases generated from the GH family 18 enzymes.²¹ This discovery has since opened a new avenue to access structurally well-defined antibody glycoforms for structural and functional studies.^{23–27} The crystal structure of Endo-S has been also determined and has provided insights into its catalytic mechanism.^{28,29}

More recently, we have generated new endoglycosynthase mutants with much broader substrate specificity from EndoS2,³⁰ an endoglycosidase from *S. pyogenes* serotype M49.³¹ Via comparison with Endo-S, the Endo-S2 enzyme is specific for serotype M49 and shares only 37% sequence identity.³¹ In addition, Endo-S2 demonstrates a substrate specificity that is much more relaxed than that of Endo-S, capable of hydrolyzing all three major types (complex, hybrid, and high mannose type) of antibody Fc N-glycans and α 1-acid glycoprotein, but Endo-S can hydrolyze only biantennary complex-type Fc Nglycans and cannot act on α 1-acid glycoprotein.^{31,32} Sequence alignment has identified a critical amino

acid residue, D184, of Endo-S2, which is equivalent to the previously reported D233 of Endo-S. Our mutational study at the critical Asp-184 residue of Endo-S2 has shown that the nature of the amino acid substituents at the critical Asp-184 residue has a significant impact on the trans glycosylation and/or the residual hydrolysis activity of the resulting mutants.³⁰ In addition, several glycosynthase mutants of Endo-S2 were found to be more active than their Endo-S counterparts. The interesting results for Endo-S2 raised the question of whether additional D233 mutants derived from Endo-S could possess further improved trans glycosylation efficiency for antibody glycan remodeling. More importantly, no mechanistic studies, such as kinetic analysis, have been performed on Endo-S and Endo-S2 mutants to date to help understand the general catalytic mechanism of endoglycosidases and glycosynthase mutants.

We report in this paper a systematic mutagenesis study at Asp-233 of Endo-S and an evaluation of the catalytic activities of the mutants. We found that substitution of Asp-233 with 19 other canonical amino acids led to significantly distinct effects on the hydrolysis and transglycosylation activities of Endo-S, with the D233M mutant showing the highest overall catalytic efficiency, defined by the transglycosylation/hydrolysis ratio. Kinetic studies of the D233M mutant of Endo-S, as well as the previously identified glycosynthase mutant D184M of Endo-S2, indicated that the enhanced overall catalytic efficacy of the Asp-to-Met mutants over that of the Asp-to-Ala mutants could be attributed mainly to two factors: the increased turnover for the glycan oxazoline donor substrate and the enhanced affinity for the antibody substrate. These findings provide a plausible mechanistic explanation for the enhanced catalytic activity of Endo-S D233M and Endo-S2 D184M mutants over other mutants.

MATERIALS AND METHODS

Materials

The monoclonal antibody, rituximab, was purchased from Genentech Inc. (South San Francisco, CA). The sialylated complex-type glycan oxazolines were chemically synthesized according to the previously published procedure.³³ Wild-type Endo-S/S2 and the mutants were expressed and purified according to protocols from our previous studies.²¹

Site-Directed Mutagenesis, Expression, and Purification of Recombinant Endo-S and Its Mutants

The pGEX plasmid vector encoding wild-type Endo-S was a gift from M. Collin (Lund University, Lund, Sweden). The Endo-S mutants were generated using the Q5 Site-Directed Mutagenesis Kit (NEB) by the manufacturer's instructions. For systematic mutagenesis at Asp-233, the following two degenerate primers were used: 5'-CGTAAATTCGTGCTCAATNNNAATATCTAGTCCATCGACACCACGATCAGTT-3' (forward) and 5'-AACTGATCGTGGTGTCTGATGGACTAGATATTNNN-ATTGAGCACGAATTACG-3' (reverse). Mutations were verified by DNA sequencing. The plasmid DNA encoding the wild-type and mutant Endo-S genes was transformed in *Escherichia coli* BL21 (DE3) cells for overexpression.

For simultaneous purification of all 20 Endo-S enzymes, the transformed cells were grown in 20 mL of 2YT medium with 100 $\mu\text{g/mL}$ carbenicillin added. Cells were incubated at 37 °C and induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when they reached an OD₆₀₀ of 0.8–1.0. The induced cells were incubated at 20 °C overnight and harvested by centrifugation. The cell lysates were collected after being treated with bacterial cell lysis buffer (Gold Biotechnology, Inc.) following the manufacturer's instructions. The glutathione S-transferase (GST)-tagged proteins were purified using the GST Spin Column Kit (Thermo). The purified proteins were diluted in phosphate-buffered saline (PBS) at pH 7.4 after buffer exchange using Amicon ultrafiltration units (10 kDa, Millipore). The purity of EndoS proteins was shown to be >90% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the final concentration was measured using a NanoDrop 2000c instrument at an absorbance of 280 nm.

Synthesis of Fuc α 1,6GlcNAc-rituximab Using WildType Endo-S Deglycosylation

To generate deglycosylated rituximab as a substrate for transglycosylation assays, the commercial antibody was treated with wild-type Endo-S at a substrate/enzyme ratio of 1000/1 (w/w) for 30 min at 37 °C to release its native heterogeneous N-glycoforms. Complete hydrolysis of N-glycans was confirmed by liquid chromatography–mass spectrometry (LC–MS) analysis. The product GlcNAc-rituximab was subsequently purified through proteinA affinity chromatography. The deconvoluted mass (m/z) of the purified antibody corresponds well to the calculated mass of Fuc α 1,6GlcNAc-rituximab.

Liquid Chromatography–Mass Spectrometry (LC–ESI-MS) Analysis of the Antibody

The LC–MS characterization of different antibody N-glycoforms was conducted on an Exactive Plus Orbitrap instrument (Thermo Scientific). The intact antibody sample was analyzed with a Waters XBridge™ BEH300 C4 column (3.5 μm , 2.1 $\times$ 50 mm). The program includes a 9 min linear gradient from 5 to 90% MeCN containing 0.1% formic acid at a flow rate of 0.4 mL/min. The original mass data were processed for deconvolution and integration using MagTran Software (Amgen).

Quantification of the Reaction Product Using an Internal Standard and a Single-Point Normalization Factor

To monitor the reaction and to quantify the transglycosylation product, either of the following two glycan-modified antibody derivatives was used as internal standards for LC–MS analysis. One was the completely deglycosylated rituximab that was generated by treatment of rituximab with peptide-N-glycosidase F (PNGase F), which removed all Fc N-glycans and introduced an Asn-to-Asp mutation at the glycosylation site;³⁴ the other was the fully sialylated glycoform, S₂G₂F-rituximab, which was synthesized according to our previously reported procedure.³⁰ Serial dilutions containing a gradient of S₂G₂F-rituximab (0, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 μM) were mixed with a stock solution containing 0.67 μM internal standard. The relative intensity of the product and the standard peaks were analyzed by selected ion monitoring (SIM) to calculate a single-point normalization factor, following the previously reported method.³⁵ For each reaction, the amount of the reaction product could be deduced from the internal standard following the normalized mathematical algorithm.

Assays on the Specific Transglycosylation and Hydrolysis Activity of Wild-Type Endo-S and Its Mutants

The hydrolytic activity of each Endo-S mutant and wild type (0.01 mg/mL) was measured in PBS (pH 7.4, 10 μ L) at 30 °C with the synthetic biantennary sialo-complex-type rituximab (1.0 mg/mL, 6.9 μ M) as the starting material. Each reaction was terminated at 5 min, and an aliquot was taken from the reaction mixture and dissolved in 0.1% formic acid. The relative quantity of the hydrolysis product was calculated through deconvolution and integration of the selected MS peaks. The experimental details of product quantification using LC–MS are illustrated above. The transglycosylation activity of Endo-S was measured in a similar fashion. Deglycosylated rituximab (10.0 mg/mL, 69 μ M) was incubated with SCT-Oxa (1.38 mM, 20 equiv) while being catalyzed by Endo-S enzymes (0.01 mg/mL) under the same condition that was used for hydrolysis. The transglycosylation product SCT-rituximab was analyzed as mentioned above. Both functional assays were repeated twice for each mutant and the wild-type enzyme, and the average activity is recorded in the Results.

Measurements of K_M and V_{max} for SCT-oxa

Serial dilutions with a total volume of 20 μ L were prepared with a gradient of SCT-oxa concentrations and a constant deglycosylated antibody concentration mixed in PBS (pH 7.4). Each reaction was initiated by adding 1 μ L of Endo-S/S2 mutants with a concentration of 0.1 mg/mL, and the reaction was conducted at 30 °C. The concentrations of SCT-oxa were within the range of 6.25–400 μ M, whereas the concentration of the deglycosylated antibody was 20 μ M. For each reaction, three aliquots were taken at 1, 2, and 3 min, and the reactions were immediately quenched in 100 μ L of 0.1% formic acid at 4 °C. The quenched reaction samples were then characterized by ESI-MS, and the concentration of the product was calculated using the normalization factor and the internal standard, as shown above. A linear progression curve was confirmed for each reaction by sampling at multiple early time points and plotting them in the same graph, which emphasized that the V_0 measured indeed represents the initial rate of each reaction. The K_M and V_{max} were calculated by fitting the initial velocities against the SCT-Oxa concentrations using GraphPad Prism7. The experiments were conducted in triplicate to determine the error in these measurements. A control reaction without the enzyme added was set up for each reaction to confirm that non-enzymatic transfer of SCT-Oxa to the antibody did not happen, and the results obtained here indeed represent the specific transglycosylation activity of each mutant.

Measurements of K_M and V_{max} for Deglycosylated Rituximab

The K_M and V_{max} values of both Endo-S and Endo-S2 mutants for the deglycosylated antibody substrate were measured in a fashion similar to that used for those of SCT-Oxa. In the serial reactions, the antibody concentrations range from 0.313 to 20 μ M, whereas the SCT-Oxa concentration was fixed at 400 μ M. A similar control reaction was also performed for each antibody concentration to monitor the non-enzymatic side reactions.

RESULTS

Saturation Site-Directed Mutagenesis of Endo-S at the Asp-233 Site

Our previous mutational studies have identified Asp-233 as a critical residue promoting sugar oxazolinium ion intermediate formation during enzymatic catalysis and further generated two glycosynthase mutants of Endo-S, D233A and D233Q, which were capable of using glycan oxazoline for transglycosylation with diminished product hydrolysis activity.²¹ To systematically screen for more efficient glycosynthase mutants of Endo-S, we constructed a library of Endo-S mutants by replacing Asp-233 with the 19 other natural amino acids through saturation mutagenesis. The mutants were expressed and purified under the same conditions that were used for wild-type Endo-S. The yields of mutants were 15–30 mg/L, which were comparable to that of wild-type Endo-S.

Comparison of Transglycosylation and Hydrolysis Activity of the Endo-S Mutants

The hydrolytic activity of the 19 Endo-S mutants as well as the wild-type enzyme was evaluated using rituximab as the substrate, and the transglycosylation activity was studied using the complex-type sugar oxazoline and the deglycosylated rituximab as the donor and acceptor substrates, respectively (Scheme 1). The results are summarized in Table 1

Comparative analysis of the hydrolytic activities revealed that all these mutants, except D233C, showed significantly diminished hydrolysis activity on the whole antibody. Specifically, D233F, D233G, D233H, D233K, D233R, D233L, D233M, D233P, D233W, and D233Y showed <1% of the intrinsic hydrolytic activity of wild-type (WT) Endo-S, whereas D233C still retained a significant capacity (33%) to hydrolyze the antibody Fc N-glycans at Asn-297. On the other hand, the transglycosylation assay revealed that most Endo-S mutants were able to transfer a biantennary complex-type glycan oxazoline to the deglycosylated rituximab antibody, with varied efficiencies. In particular, D233C, D233E, D233G, D233M, D233N, and D233S demonstrated the most significant transglycosylation activities. However, via comparison of the overall transglycosylation efficiencies, most mutants with potent transglycosylation activities also possessed residual hydrolysis activity, a property less ideal for antibody Fc glycan remodeling. For instance, D233C demonstrated the highest activities for both hydrolysis and transglycosylation among all the mutants, but its overall efficiency for antibody glycoform synthesis (defined by the ratio of transglycosylation to hydrolysis, T/H) was found to be moderate. Interestingly, the D233M mutant retained a relatively high transglycosylation activity among the mutants with a remarkably low residual hydrolysis activity. Thus, the highest overall synthetic efficiency makes D233M the most efficient glycosynthase mutant of Endo-S for antibody glycoengineering among the mutants studied here. On the basis of the results of transglycosylation and residual hydrolysis activities of the mutants (Table 1), the D233M mutant showed an approximately 7- and 8-fold enhanced synthetic efficiency (T/H) versus those of the previously reported D233Q and D233A,²¹ respectively. To demonstrate the improved catalytic efficiency of Endo-S D233M for Fc glycosylation of the deglycosylated antibody, we compared the glycosylation activities of three selected mutants, D233M, D233Q, and D233A, with the glycan oxazoline under the same glycosylation conditions. The parallel reactions were monitored by LC–ESIMS (Figure 1). The comparison of the

time course of each reaction indicated that mutant D233M was more efficient than the previously identified mutants, D233A and D233Q.²¹ For instance, the D233M-catalyzed reaction reached 95% transformation of the antibody within 1 h, while the D233A- or D233Q-catalyzed reactions gave an only ~60% yield at 1 h under the same reaction conditions (Figure 1).

Kinetic Analysis of Endo-S D233M and Endo-S D233A

The observed difference in the catalytic activity of D233M and D233A mutants prompted us to perform a kinetic analysis of the two mutants. Using a sialylated glycan oxazoline (SCT-Oxa) as the donor and the deglycosylated rituximab as the acceptor substrate, we measured the kinetic parameters of both Endo-S mutants. A mass spectrometry-based approach with an internal standard was used to estimate product formation.^{35–37} The results are summarized in Tables 2 and 3. For the biantennary complex-type glycan oxazoline (SCT-Oxa), the catalytic efficiency of Endo-S D233M ($k_{\text{cat}}/K_M = 0.03 \text{ min}^{-1} \mu\text{M}^{-1}$) showed a 3-fold increase in comparison with that of Endo-S D233A ($k_{\text{cat}}/K_M = 0.01 \text{ min}^{-1} \mu\text{M}^{-1}$) (Table 2 and Figure 2a). The apparent increase in the k_{cat}/K_M of Endo-S D233M versus that of D233A was a result of an approximately 4-fold increase in the rate of turnover (as measured by k_{cat}) and a <2-fold reduced affinity as estimated by the K_M value, as a higher K_M value is indicative of a lower substrate affinity. These results suggested that substitution with a methionine instead of an alanine at Asp-233 enhanced the enzymatic turnover number with a moderate decrease in substrate affinity for the glycan oxazoline substrate.

For the glycosyl acceptor substrate, the deglycosylated antibody, the Endo-S D233M mutant showed a substrate affinity much higher than that of the D233A mutant. This was clearly supported by the Michaelis–Menten curves of the two mutants (Figure 2b), which demonstrated that Endo-S D233M was much easier to saturate with the antibody substrate than D233A was. The K_M value of D233M for the deglycosylated antibody was $30 \mu\text{M}$, while the K_M of D233A for the deglycosylated antibody was $>200 \mu\text{M}$ (Table 3). On the other hand, the catalytic turnover number of Endo-S D233A (k_{cat}) for the antibody substrate could not be accurately measured because the concentration of the deglycosylated antibody required to saturate the enzyme was difficult to achieve.

Kinetic Analysis of Endo-S2 D184M and Endo-S2 D184A

Endo-S2 is another endoglycosidase from *S. pyogenes* serotype M49 that shows a substrate specificity much broader than that of Endo-S for antibody deglycosylation.³¹ Our recent study of the mutations at the critical Asp-184 residue has identified the D184M mutant as the most efficient glycosynthase mutant within the systematic library of EndoS2 variants developed by saturation mutagenesis.³⁰ To evaluate the nature of the enhanced catalytic efficiency, we measured the kinetic parameters of D184M and D184A mutants of Endo-S2. The results revealed highly similar patterns between the Endo-S and Endo-S2 mutants (Tables 4 and 5).

For the sugar oxazoline, the Endo-S2 methionine mutation significantly enhanced its catalytic efficiency (k_{cat}/K_M) in comparison with that seen for the alanine mutation, and an approximately 10-fold increase in k_{cat}/K_M was observed for the D184M mutant over the

D184A mutant of Endo-S2 (Table 4 and Figure 3a). In analogy to Endo-S, the increased $k_{\text{cat}}/K_{\text{M}}$ value for D184M mutant was caused by a >15-fold increase in the catalytic turnover number (k_{cat}) and a <2-fold decrease in the substrate affinity (K_{M}) for the glycan oxazoline substrate.

For the deglycosylated rituximab, like the Endo-S D233M mutant, the Endo-S2 D184M mutant also showed an affinity for the antibody substrate significantly higher than that of the D184A mutant. The K_{M} value of Endo-S2 D184A was estimated to be >20 times higher than that of the Endo-S2 D184M mutant. Similar to the case of Endo-S, Michaelis–Menten curves also indicated that the Endo-S2 D184A mutant showed no sign of saturation within the range of antibody concentrations tested. Therefore, the catalytic turnover number of the Endo-S2 D184A mutant also could not be accurately measured because of the difficulty of reaching a saturated concentration of the substrate (Table 5 and Figure 3b).

Interestingly, for both the sugar oxazoline and the antibody substrate, Endo-S2 D184M demonstrated a catalytic turnover number significantly higher than that of Endo-S D233M, whereas the substrate affinity for both substrates proved to be comparable between the two mutants. Therefore, the overall catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of Endo-S2 D184M was found to be higher than that of Endo-S D233M.

DISCUSSION

We have previously identified two glycosynthase mutants, D233A and D233Q of Endo-S, which can transfer glycan oxazoline to the Fc-deglycosylated antibody to form homogeneous antibody glycoforms.²¹ More recently, we have identified new glycosynthase mutants from Endo-S2 by mutation at the critical Asp-184 residue, which show broader substrate specificity and distinct efficiency for antibody Fc glycan remodeling.³⁰ However, a systematic mutagenesis on the prototype enzyme Endo-S at the critical Asp-233 site had not been performed, and mechanistic insight into the different catalytic activities of these mutants is currently lacking.

In this work, we performed a systematic mutagenesis of Endo-S at Asp-233 and examined the residual hydrolysis activities and transglycosylation activities of the mutants. The results revealed several novel glycosynthase mutants of Endo-S such as the D233M mutant that demonstrated a transglycosylation efficiency that was greatly enhanced compared to that of the previously identified Endo-S mutants. The kinetic analysis clearly indicated that the enhanced catalytic efficiency of the Endo-S D233M mutant versus that of the D233A mutant could be attributed to mainly two factors: the increased turnover for the glycan oxazoline donor substrate and the enhanced affinity for the antibody substrate. This was found to be true also for the much higher catalytic efficiency of the Endo-S2 D184M mutant versus that of the Endo-S2 D184A mutant. Nevertheless, the molecular basis behind the observed enhancement of catalytic efficiency by the Asp-to-Met mutation, i.e., the increased turnover of the glycan oxazoline substrate and the enhanced affinity for the antibody substrate, remains to be elucidated.

The crystal structure of Endo-S was first reported in 2014, and more recently, the structure of full-length Endo-S in complex with a complex-type N-glycan was determined.^{28,29} In the latter study, a catalytically inactive variant of Endo-S, in which both the general acid/base residue E235 and the critical residue D233 were replaced with leucine and alanine, respectively, was created to abolish the enzyme's hydrolytic activity on the N-glycan,²⁹ but unfortunately, the crystal structures of Endo-S and its complex with the ground state N-glycan could not precisely reflect how the glycan oxazoline substrate would be recognized at the catalytic site. One potential solution is to determine the structure of the enzyme in complex with a stable, nonhydrolyzable glycan oxazoline analogue, the glycan thiazoline, which we have recently synthesized.³⁸ However, it should be pointed out that even if a structure of the enzyme--glycan oxazoline analogue complex can be determined to show the enzyme-substrate recognition, it might not be able to provide much information about the enhanced turnover observed for the Asp-to-Met mutations. On the other hand, a future structural study of Endo-S D233M or Endo-S2 D184M in complex with a deglycosylated Fc domain could provide valuable information about how the methionine mutants could significantly enhance the affinity for the antibody Fc domain, as observed from the kinetic analysis presented here.

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Figure 1. Comparison of the transglycosylation reaction of Endo-S D233M and previously reported Endo-S D233Q and D233A using SCT-Oxa as the substrate. The reaction was conducted by incubating the deglycosylated antibody and SCT-Oxa with a fixed concentration of each enzyme (0.05 mg/mL). The molar ratio of antibody to oxazoline was controlled at 1/20. The data sets presented here are representative of three independent experiments.

Figure 2. Michaelis–Menten plots of Endo-S mutants (D233M and D233A) for the two substrates. (a) For the glycosyl donor, a gradient of SCTOxa (from 6.25 to 400 μM) was tested with a constant concentration of deglycosylated rituximab (20 μM). (b) For the glycosyl acceptor, a serial dilution of rituximab (from 0.32 to 20 μM) was tested with 400 μM SCT-Oxa. For both Endo-S D233M and D233A, the enzyme concentration was controlled at 0.01 mg/mL (0.067 μM). The data sets presented here are representative of three independent experiments.

Figure 3. Michaelis–Menten plots of Endo-S2 mutants (D184M and D184A) for the substrates. (a) For the glycosyl donor, a gradient of SCT-Oxa (from 6.25 to 400 μM) was tested with a constant concentration of deglycosylated rituximab (20 μM). (b) For the glycosyl acceptor, a serial dilution of rituximab (from 0.32 to 20 μM) was tested with 400 μM SCT-Oxa. For both Endo-S2 D184M and D184A, the enzyme concentration was controlled at 0.01 mg/mL (0.067 μM). The data sets presented here are representative of three independent experiments.

Scheme 1.

Hydrolysis and Transglycosylation Reactions by Endo-S and Its Mutants Using Commercial Antibody Rituximab as the Substrate

Table 1.Comparison of the Specific Hydrolysis and Transglycosylation Activities of Endo-S and Its D233 Mutants^a

Endo-S mutant	specific hydrolysis activity ($\times 100 \mu\text{mol min}^{-1} \text{mg}^{-1}$)	transglycosylation activity ($\times 100 \mu\text{mol min}^{-1} \text{mg}^{-1}$)	transglycosylation/hydrolysis ratio T/H
wild type	2.77 $\pm$ 0.64	25.9 $\pm$ 4.7	9.35
D233A	0.06 $\pm$ 0.01	1.06 $\pm$ 0.2	17.7
D233C	0.92 $\pm$ 0.16	21.3 $\pm$ 3.5	23.1
D233E	0.36 $\pm$ 0.09	9.45 $\pm$ 1.6	26.3
D233F	0.02 $\pm$ 0.01	0.16 $\pm$ 0.04	8.00
D233G	0.25 $\pm$ 0.04	3.13 $\pm$ 1.2	12.5
D233H	0.01 $\pm$ 0.01	0.00	0.00
D233K	0.01 $\pm$ 0.01	0.00	0.00
D233R	0.01 $\pm$ 0.01	0.00	0.00
D233I	0.04 $\pm$ 0.01	0.14 $\pm$ 0.05	3.50
D233L	0.02 $\pm$ 0.01	0.14 $\pm$ 0.03	7.00
D233M	0.02 $\pm$ 0.01	2.71 $\pm$ 0.4	136
D233N	0.43 $\pm$ 0.12	2.96 $\pm$ 1.3	6.88
D233P	0.02 $\pm$ 0.01	0.00	0.00
D233Q	0.05 $\pm$ 0.01	0.98 $\pm$ 0.2	19.6
D233S	0.36 $\pm$ 0.01	3.65 $\pm$ 0.6	10.1
D233T	0.21 $\pm$ 0.03	1.79 $\pm$ 0.3	8.50
D233V	0.21 $\pm$ 0.06	0.25 $\pm$ 0.08	1.20
D233W	0.01 $\pm$ 0.01	0.00	0.00
D233Y	0.01 $\pm$ 0.01	0.26 $\pm$ 0.1	26.0

^aThe hydrolysis and transglycosylation activities were measured by LC-ESI-MS monitoring of the reactions. The specific hydrolysis activity was assayed by incubating the synthetic biantennary complex-type rituximab with wild-type Endo-S or its different mutants (0.01 mg/mL) in PBS (pH 7.4) at 30 °C. To test the specific transglycosylation activity of each enzyme, the deglycosylated rituximab was used as the acceptor and SCT glycan oxazoline as the donor substrate with a final concentration of enzymes of 0.01 mg/mL. The molar ratio of glycosyl acceptor to donor was 1/20. The experiments were conducted in duplicate.

Table 2.

Kinetic Parameters of Endo-S Mutants D233M and D233A for the Glycan Oxazoline

enzyme	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)
Endo-S D233M	5.0 $\pm$ 0.9	195 $\pm$ 7.2	0.03
Endo-S D233A	1.3 $\pm$ 0.2	87.9 $\pm$ 9.1	0.01

Table 3.

Kinetic Parameters of Endo-S Mutants D233M and D233A for Rituximab

enzyme	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)
Endo-S D233M	5.5 ± 1.9	30.2 ± 7.2	0.18
Endo-S D233A	<i>a</i>	>200	<i>a</i>

^aThe catalytic turnover number of Endo-S D233A could not be accurately determined because the concentration required for antibody substrate saturation was not reached in the experiments.

Table 4.

Kinetic Parameters of Endo-S2 Mutants D184M and D184A for the Glycan Oxazoline

enzyme	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)
Endo-S2 D184M	168 $\pm$ 21	229 $\pm$ 35	0.73
Endo-S2 D184A	10.2 $\pm$ 2.4	121 $\pm$ 17	0.08

Table 5.

Kinetic Parameters of Endo-S2 Mutants D184M and D184A for Rituximab

enzyme	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)
Endo-S2 D184M	29.8 ± 9.3	32.6 ± 6.7	0.91
Endo-S2 D184A	<i>a</i>	>680	<i>a</i>

^aThe catalytic turnover number of Endo-S2 D184A could not be accurately determined because the concentration required for antibody substrate saturation was not reached in the experiments.